Lab note of target 1-3

Construction of Circuits

Parts from iGEM took kit (8/11~8/27)

Out Line:

For each part to be used in testing circuit:

BBa_J23100 , BBa_E1010, BBa_B0015

After transforming those plasmid into our competent cell, DH5a, we would take 3 steps to examine if we got the right product:

1. colony PCR and electrophoresis : Directly picking up colonies on designated antibiotic plate and do colony PCR with iGEM provided primers(VF2&VR), and then do electrophoresis to check the length of plasmid to confirm if the kit had been correctly transformed into E.coli.
2. plamid PCR and electrophoresis : Colony PCR may not end up coping the right place, for there are extra DNA in the cell that is not the one of our interest. Therefore, we also check the lenth of the plasmid we which we transformed.
3. digestion and electrophoresis : After checking the lenth, we use EcoR1/Spe1 and Xba1/Pst1, and electrophoresis, to see if our constructed plasmid can be successfully digested.

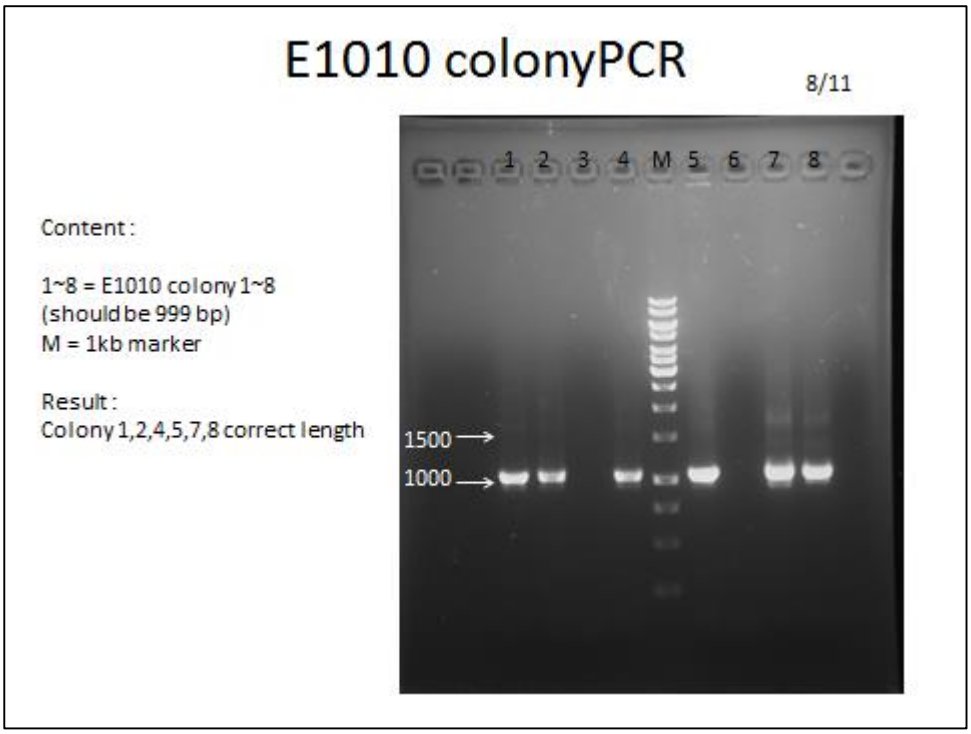
Process:

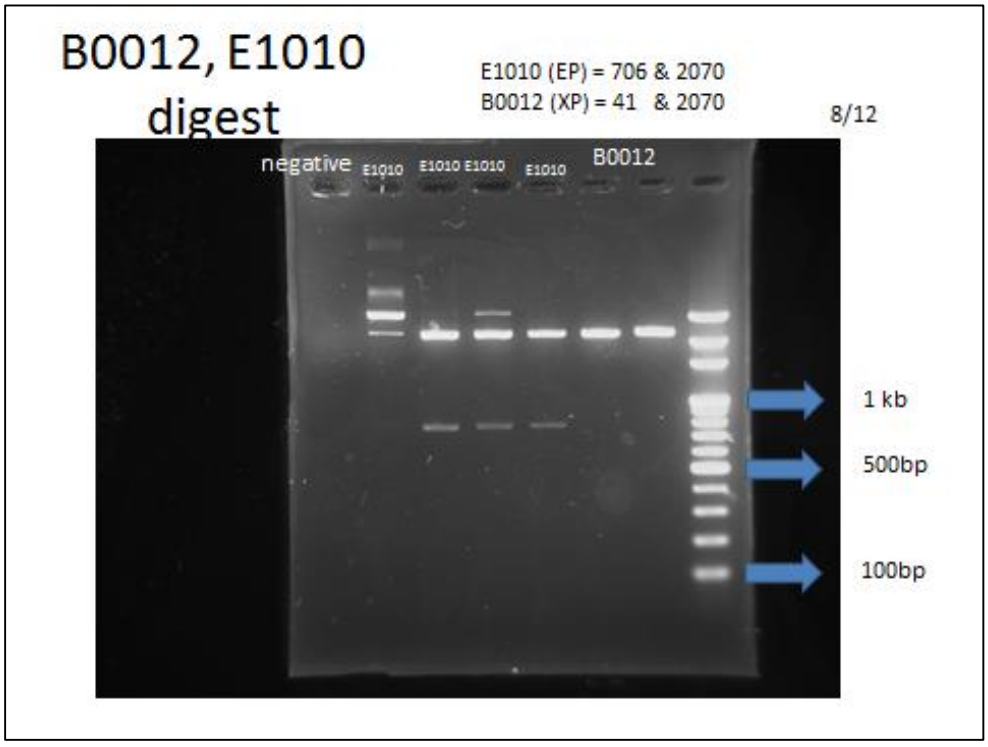
We encounter several difficulties during the process:

1. In the transformation step, we couldn't get transformation with the right PCR band at first. Even thought they do grow on the antibiotic plate, electrophoresis result doesn't show any band. Therefore, we tried different ways trying to solve these problem.
   First, we thought it was PCR problem, therefore, we tried colony PCR, however, it came out that it wasn't the case.
   So we improve our transformation protocol. That is, after 1.5 hour of recovery in 37C incubator in 200ml LB, we centrifuge in 3.4 rpm for 20 seconds, and discard 150ml supernatant. At the end, we result in putting concentrated transformed E.coli into our antibiotic plate. This way, we sequentially transformed several kits successfully.
2. In the checking step, we've have contaminated electrophoresis result after colony PCR. That is, we got band in the negative well. To find out the

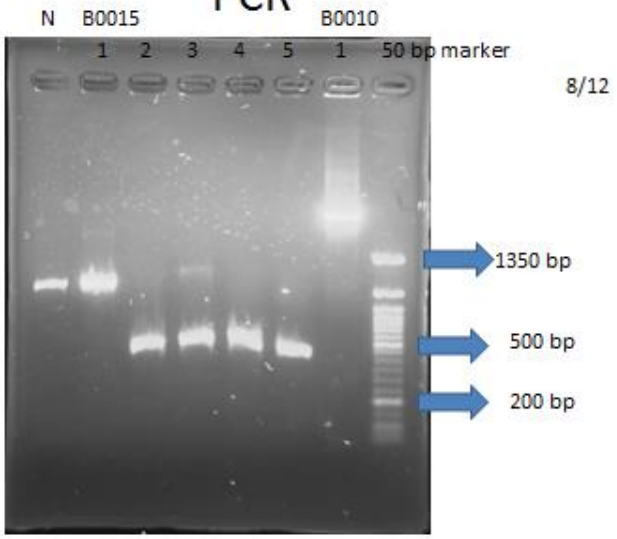
ingredient causing contamination, we do several negative control changing different packing of ingredients. And find the contamination of our 10X DreamTaq Buffer.

Result :





B0015(443 bp) B0010(218bp) colony PCR

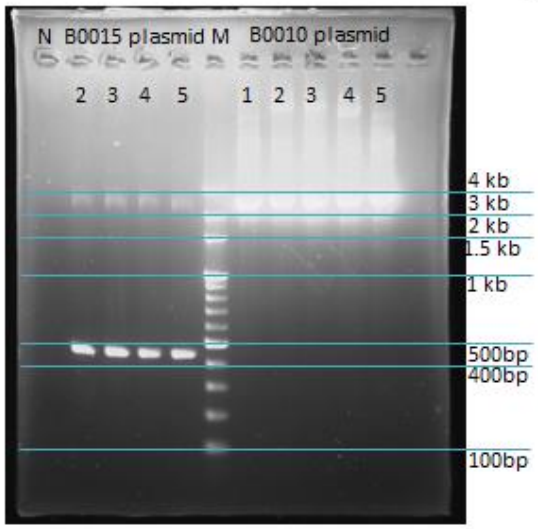


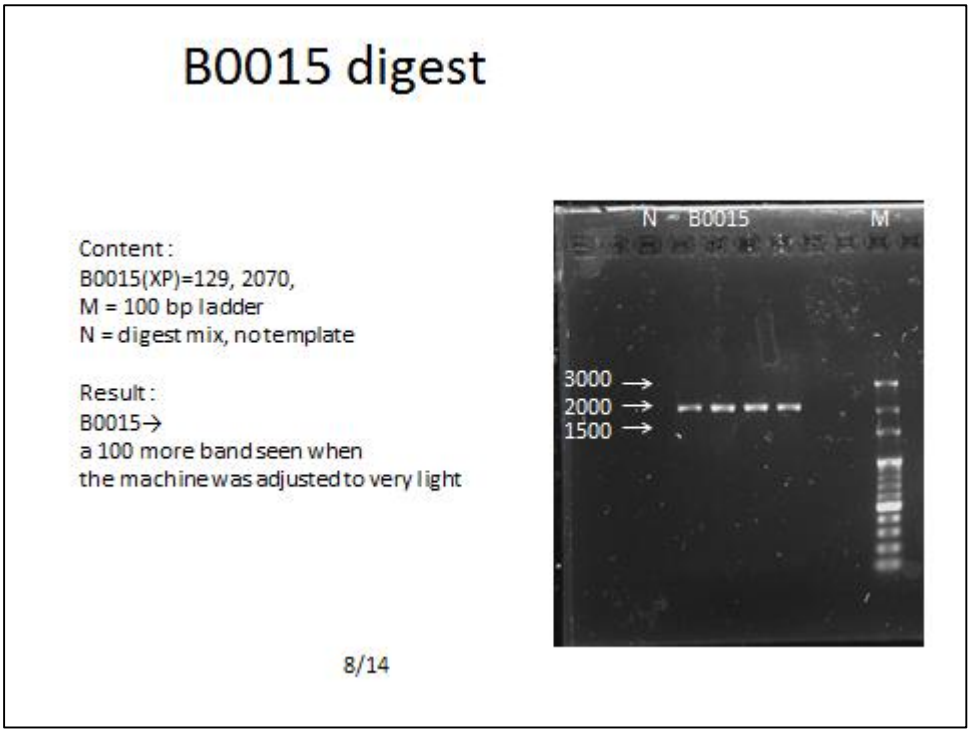
8/12

B0015,B0010 plasmid PCR

8/13

N = PCR mix
B0015 = 443 bp
M = 100 bp ladder
B0010 = 218 bp





Parts that needed extra PCR(8/18~9/22)

Outline

Two parts needed extra primer in order to get them:
yebF, RFP, and M102 orf19

And for each part, we do two kinds of PCR , one using Dream Tag, only to check the lengh to ensure we got our part. And then we used KOD enzyme, together with magnesium cation, to check the lengh, furthermore, do gel extraction to get the part. That's because KOD enzyme leads a more accurate PCR.

- For yebF, we designed primers to get the sequence we want yebF (its own ribosomal binding site included) from Escherichia coli MG1655.
  Front Primer : tttctagagGGAGAAAAACATGAAAAAAAGAGGGGCGTT
  (with Xbal cutting site)
  Reverse Primer : attctgcagcggccgctactagtACGCCGCTGATATTCCGCCA
  (with Spel and Pst1 cutting site)
  The PCR product should be 389bp.
- Because the need of recombinant protein, the RFP downstream of yebF need to be specially designed in order to leave scar with multiple of 3. E1010 was used as template, and primers with specially designed cutting site is made:
  Front Primer: tttctagaGCTTCCTCCTCCGAAGACGTTATC
  And normal iGEM reverse primer, VR

- To deprive endolysin from Strepptococcus Mutans, following primers are designed. Extra considerations needed to be made in order to produce recombinant protein. Also, due to the Spel site occurred in orginal M102 open reading from of the phage, extra primers making point mutation should be made:

  Endolysin_Front Primer : tttctagaACCTCGCTAAAAAAAGGT

  (with Xbal cutting site)

  Endolysin_Reverse Primer : attctgcagcggccgctactagtaTTATTTTCCTTTGTTAATTACTGC

  (with Spel & Pst1 cutting site)

  Endolysin_point mutation_Front Primer : CAAGTTGCAACAAGCGATTGCATTG

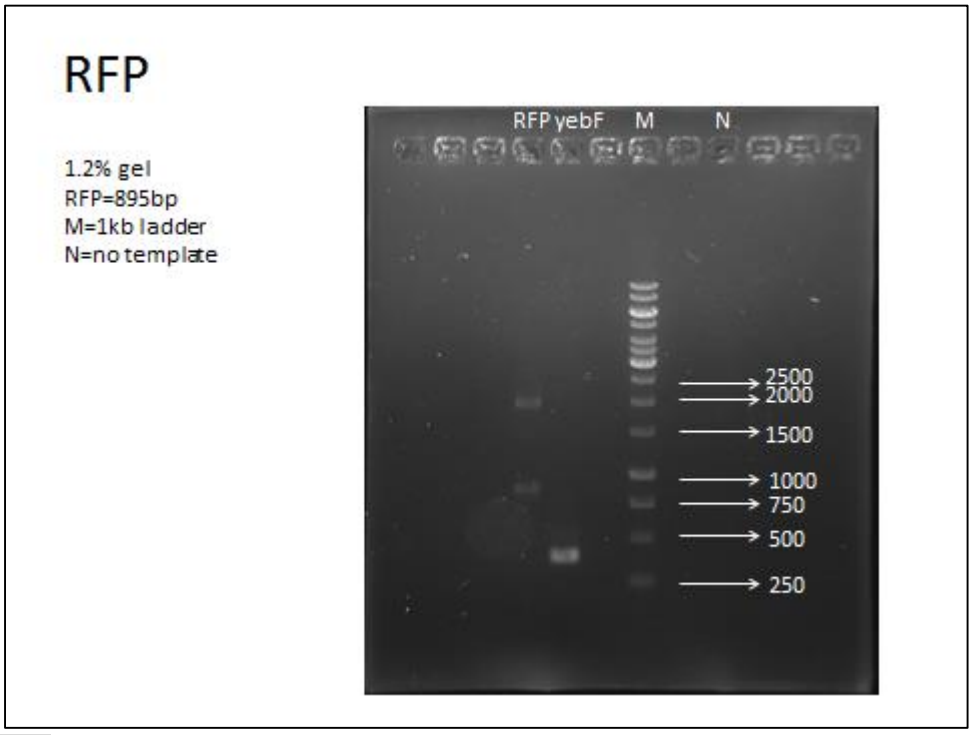
  Endolysin point mutation_Reverse Primer : CAATGCAATCGCTTGTTGCAACTTG

  1. By using Endolysin_Front Primer & Endolysin_mutation_reverse primer, we would get a product of 175 bp.
  2. And Endolysin_mutation_front primer & Endolysin reverse primer would result in a 696bp- PCR product.
  3. The gel extraction product of the previous steps together with Endolysin_front primer & Endolysin_reverse primer could help us get the final product.

Process

While doing RFP PCR, we got extra band.

By adjusting PCR program, we finally got correct band.

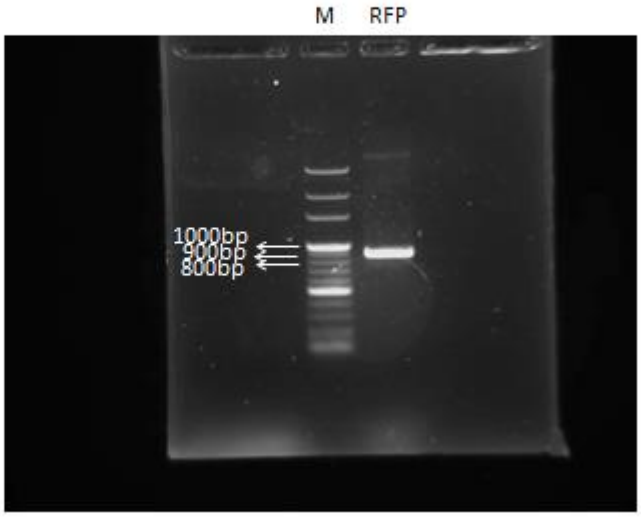


Result:

The yebF came out pretty well, so after running KOD PCR and pre-check the length, we did gel extraction to get yebF. So is the specially designed RFP.

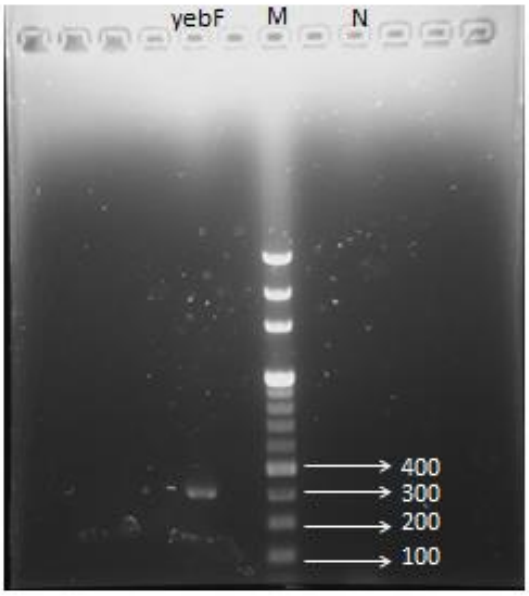
RFP

RFP=895 bp
M=100 bp marker



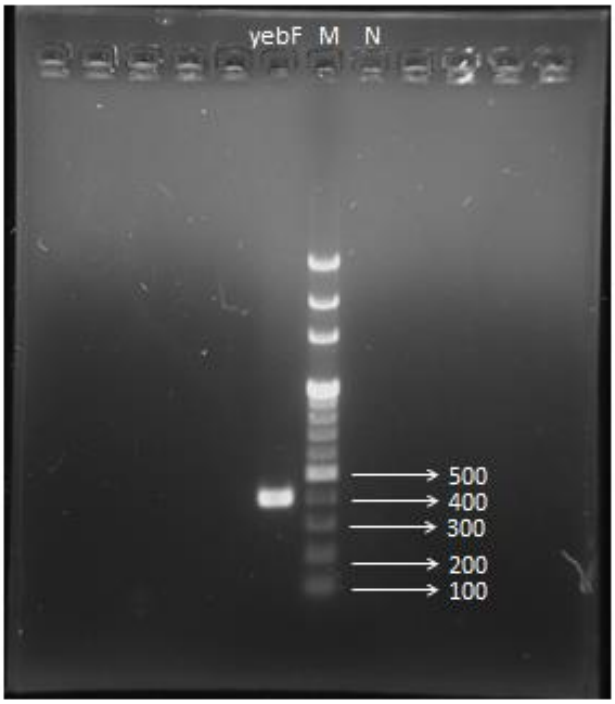
yebF

yebF 396bp
M=100 bp marker
N=no template



yebF_KOD

1.5% gel
yebF=396bp
M=100 bp maker
N=no template



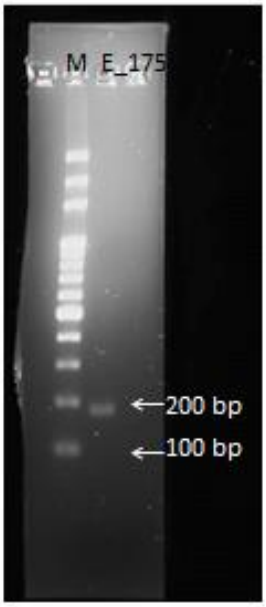
Endolysin_175bp

- Content :

E_175= Endolysin PCR product
-Primers: Endo_FP&
Endo_mut_RP
-Program : 55
- should be 175 bp
M = 100 bp marker

- Result :

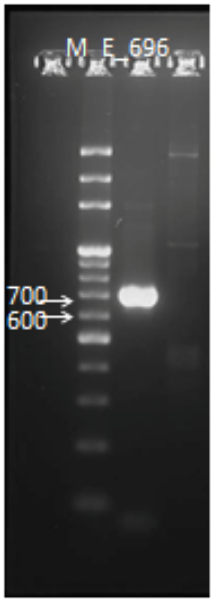
Correct length

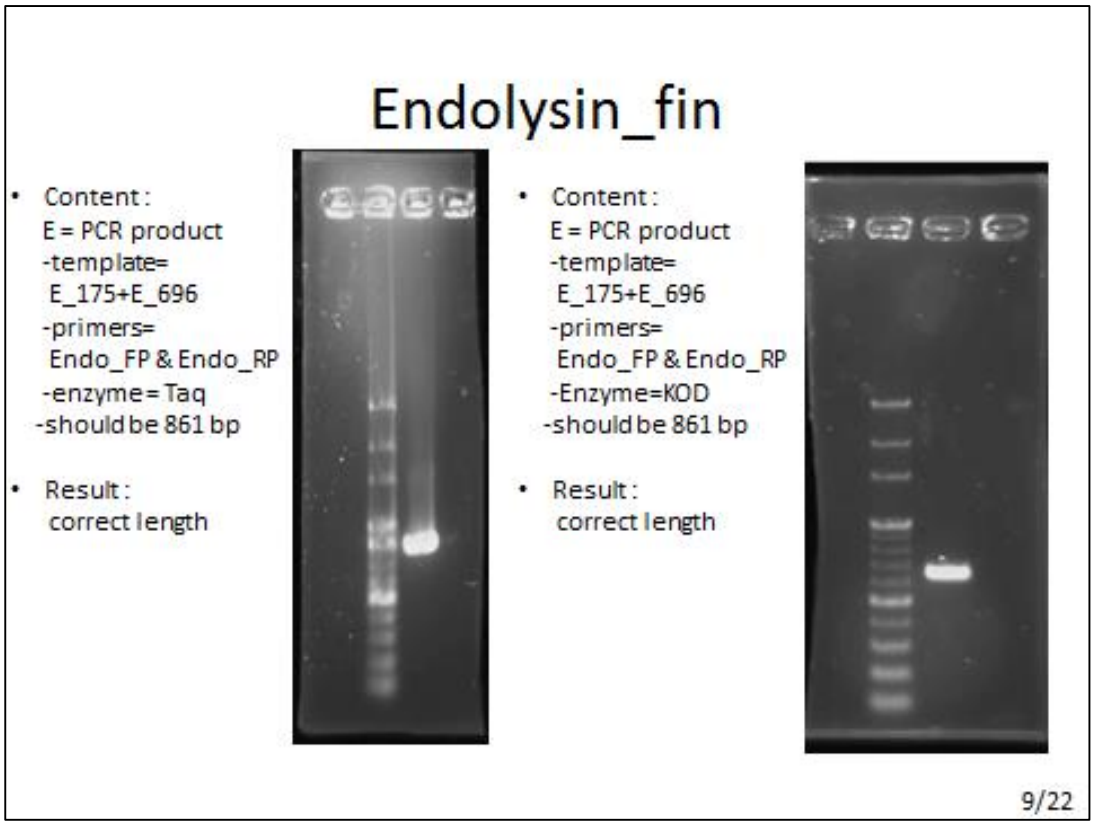


Endolysin_696bp

- Content :
  E_696 = PCR product
  -template=M!02 DNA
  -primers=Endo_RP & Endo_mut_FP
  - Program=700~1000TD
  - should be 696 bp
  M = 100 bp marker

- Result :
  Correct length





Construction of the circuit:(8/28~10/5)

Outline:

Circuits needed to be constructed:

J23119+RBS-yebF+RFP+B0015
J23119+B0034+RFP+B0015

J23119+RBS-yebF+B0015
J23119+RBS-yebF+endolysin+B0015

By repeatedly using back insert, we tried to achieve the final construction.

We planned to constructed our first circuit by following steps

J23119(SP)+yebF(XP)
J23119+RBS-yebF(SP)+RFP(XP)
J23119+yebF+RFP(SP)+B0015(XP)
J23119(SP)+yebF(XP)
J23119+RBS-yebF(SP)+endolysin(XP)
J23119+RBS-yebF+endolysin(SP)+B0015(XP)

Due to the problems of the plasmid, we do the following design as the result:

J23100
J23100+RBS-yebF+RFP+B0015

J23100+RBS-yebF+B0015
J23100+RBS-yebF+endolysin+B0015

To finish the design, and being admitted to part submission(pSB1C3) simultaneously, we combined front, and back insert to help us achieve our goal.

J23100(SP)+yebF(XP)
J23100+RBS-yebF(SP)+RFP(XP)
J23100+RBS-yebF+RFP(ES)+B0015(EX)

J23100(SP)+yebF(XP)
J23100+RBS-yebF(SP)+endolysin (XP)
J23100+RBS-yebF+endolysin(ES)+B0015(EX)

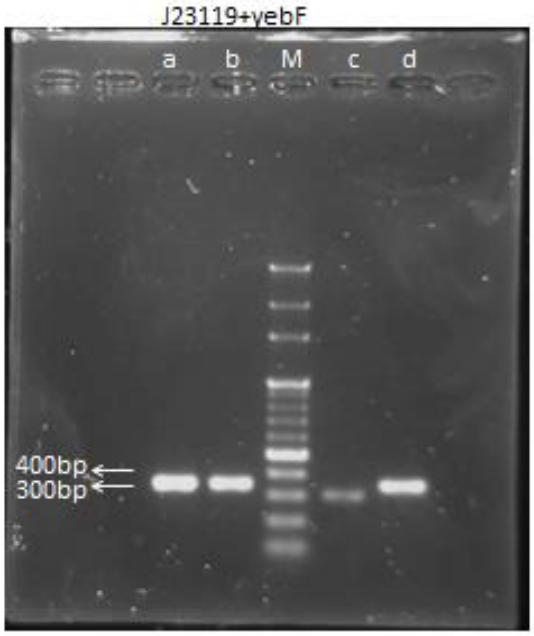
Process:

During circuit construction, we encountered several problems:
First, we found out that the enzyme that we own are from different companies. Our digestion enzyme Pst1 is from Fast Digestion, requiring FD buffer, while the Spel enzyme is from NEB, requiring the addition of NEB buffer 4 and BSA, or smart cut buffer.
Also, because that one of the part result from cutting is so small that it's invisible in electrophoresis, we can only check if our product had been successfully cut by transformation. We also did electrophoresis, though, to check if the band would be good enough to do extraction. Finally, by doing checking transformation product after ligation, we can fully sure that we had the right digestion cut.
For our first ligation, J23119+yebF, its result shows that using FD buffer is absolutely impossible if we want to digest with Spel and Pst1. As shown on the graph, it might just stick back to the original plasmid.

J23119+yebF

Primers: VR+VF2
Predicted lengh: 564 bp
M = 100 bp marker

PS. J23119 PCR with VR & VF2
Predicted size = 348 bp



In our later attempts trying to ligate J23119+yebF, we got few more failed results: Including that there are no colonies on the transformation plate, resulting incorrect bands, and more problems.

And here's the thing, while using SpeI and Pst1 to digest J23119, it would result in 18 bp and 2087bp , which the 18 bp is almost invisible. Also, using Xbal and Pst1 to digest the KOD PCR product of yebF would show insignificant result that can not be confirmed. Therefore, it resulted in too much variebles. Trying to decrease some variables, we decided to change strategies. Using J23100 as a promoter by cutting up Spel and Pst1 would result in 2096 bp and 887 bp, which could help us clear up at least one variable. From the digestion electrophoresis, we extract both fragments out, hoping to do 2 construct simultaneously. And, more focus on the result of digested J23100 for it's convenience of trouble shooting(J23119+ & J23100+yebF).
This is our result:

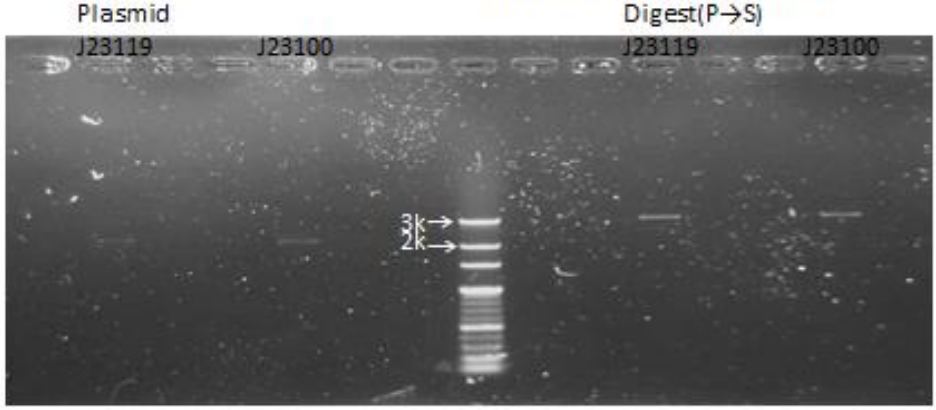
J23100 (S+P)_cutsmart

- Content :
- J23100(SP) from colony C & D
  Using SpeI(NEB) & Pst1 (FD) together, using cutsmart 10X buffer
  37C water bath for 3hr
  -should be 887 & 2096 bp
- Positive control= J23100 plasmid
  - should be 2983 bp
- M=100 bp marker
- Result:
- Digested product showing only one band with a approximate 3000bp length→might only one cutting site cut

J23119 & J23100 (Pst1→SpeI)

- Content :
- J23119 & J23100
  -First cut with Pst1
  -Ater gel extraction, digest with SpeI
  -Each digestion 37C water bath for 3hr
  -should be 887 & 2096 bp

- Positive control= J23100 plasmid
  - should be 2983 bp
- M=100 bp marker
- Result:
- Digested product showing only one band with a approximate 3000bp length→might only one cutting site cut



9/12

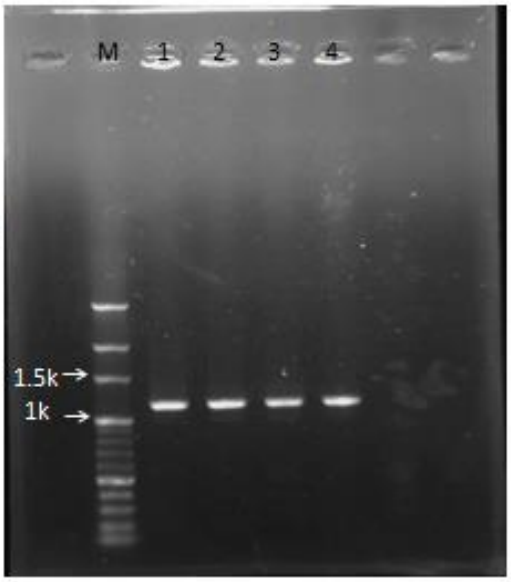
All of the results show that there is only one site being cut. We suspected that there might be some problem of our previous second time plate.

While we didn't have bacterial glycerol stock, we decided to re-transform it from the toolkit. However, we found that our J23119 toolkit had been lost, therefore, we

decided that J23100 would be our substitute promoter.

J23100(colony PCR)

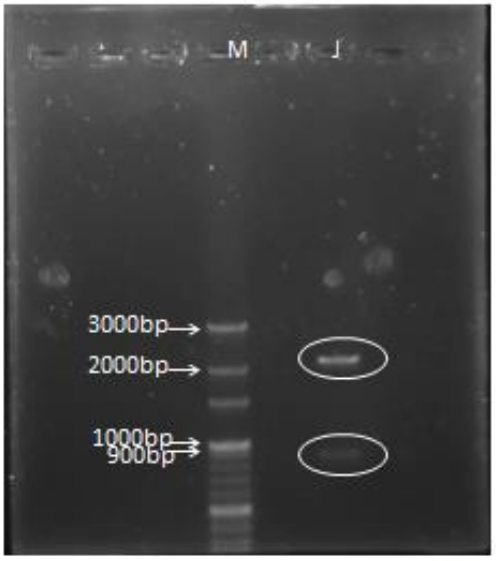
- Content :
  -1~4=J23100 PCR product from colony 1~4
  -M = 100 bp marker
- Result :
  All showing band with correct size



9/14

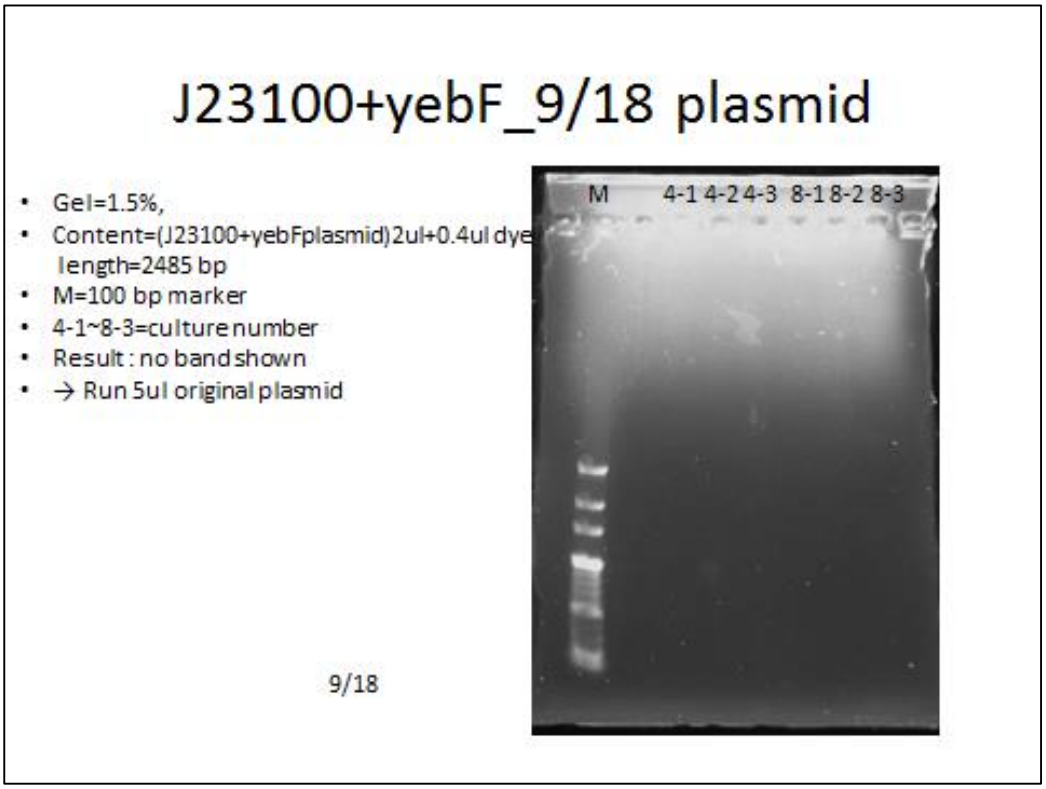
J23100 (SpeI&Pst1)

- Content :
  -J = J23100 cut with SpeI and Pst1, using Fast Digestion buffer (should be 887bp & 2096 bp)
  -M = 100 bp marker
- Result :
  Expected length



9/15

The next problem we encounter is that we can't see J23100+RBS-yebF under electrophoresis result for a while. After changing the kit, we got enough plasmid to be seen on electrophoresis graph, so do proceeding digestion product.

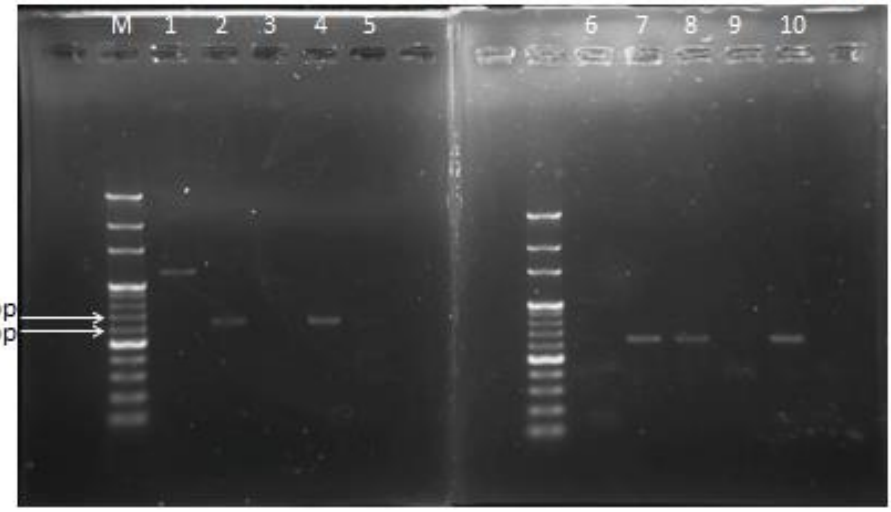


Result:

J23100+yebF_colony PCR

- Gel=1.5%,
- Product=643 bp
- M=100 bp marker , 1~10=colony number
- Primer=VF2+VR, Program=55
- Result : 2,4,7,8,10,should be correct

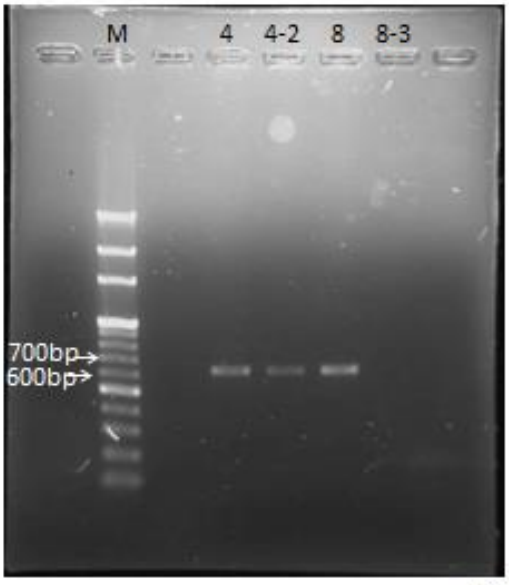
9/17



J23100+yebF_plasmid PCR

- Gel=1.5%,
- Product=643 bp
- M=100 bp marker
- 4 ,4-2,8,8-3=culture number
- Primer=VF2+VR, Program=55
- Result : correct, except 8-3

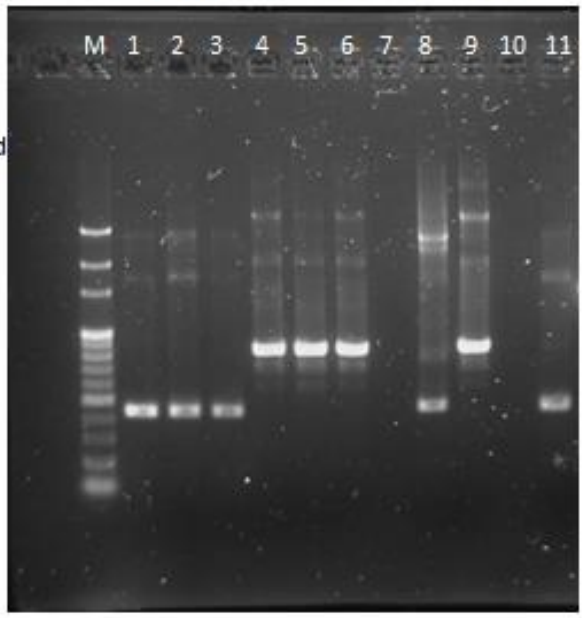
→ may be the error existed while adding mix



9/19

J23100+yebF+B0015(plasmid PCR)

- Content :
  1~11=PCR product
  -template=J23100+yebF+B0015 plasmid from colony 1~11
  -primers=VF2+VR
  -program=TD2000
  -should be=854bp
  M = 100 bp marker
- Result :
  colony 4,5,6,9 length correct

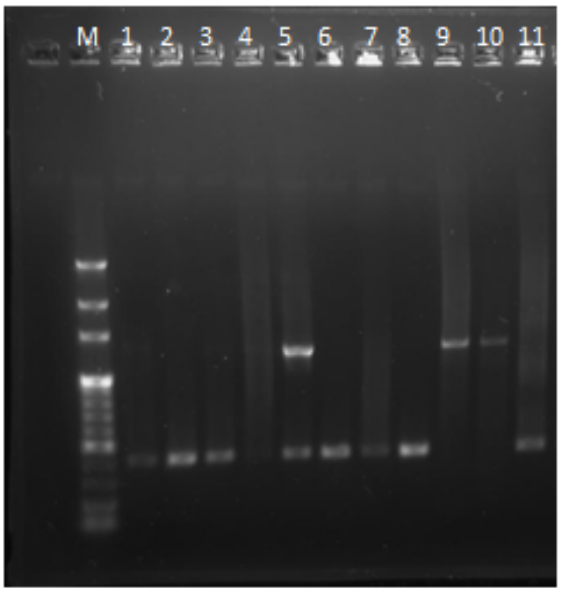


10/3

J23100+yebF+RFP(plasmid PCR)

- Content :
  1~11= PCR product
  -template=J23100+yebF plasmid from colony 1~11
  -primers = VF2+VR
  -program = TD2000
  -should be 1369bp
  M = 100 bp marker

- Result :
  contamination
  colony 5,9 correct length

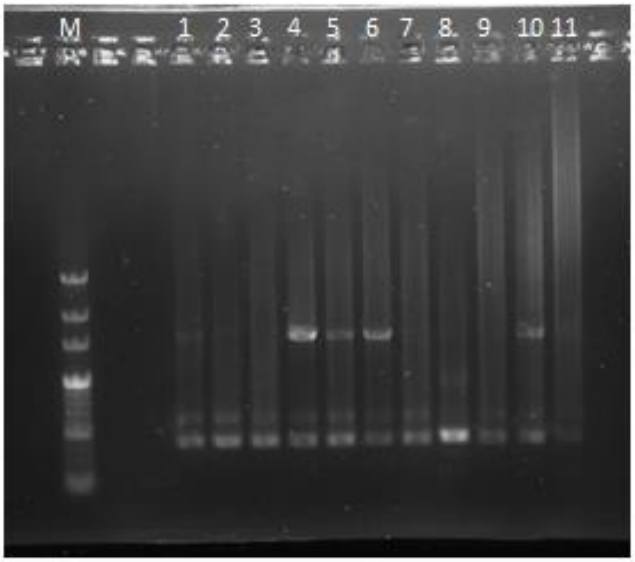


9/28

J23100+yebF+RFP+B0015(plasmid PCR)

- Content :
  1~11= PCR product
  -template=
  J23100+yebF+RFP+B0015
  plasmid from colony 1~11
  -primers = VF2+VR
  -program = TD2000
  -should be 1566
  M = 100 bp marker

- Result :
  contamination
  colony 4,5,6,10 correct

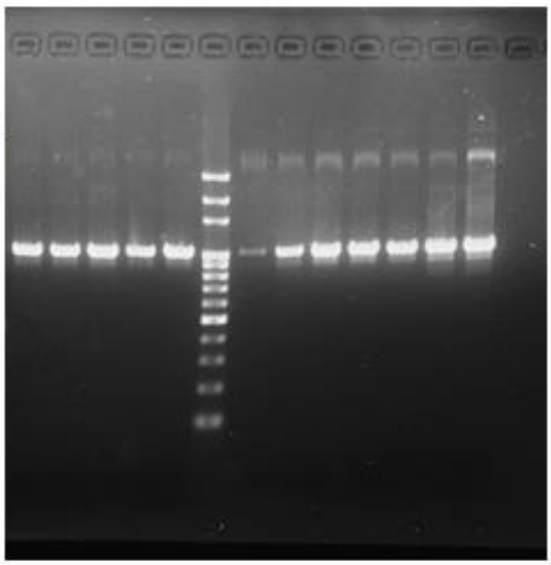


9/30

J23100+yebF+endolysin(plasmid PCR)

Content :
1~12=PCR product from colony1~12
-template=J23100+yebF+endolysin
-primer=VF2+VR
-program=700~1000TD
-should be 1434bp
M = 100 bp marker

Result :
All of the colonies(1~12) are correct



10/2

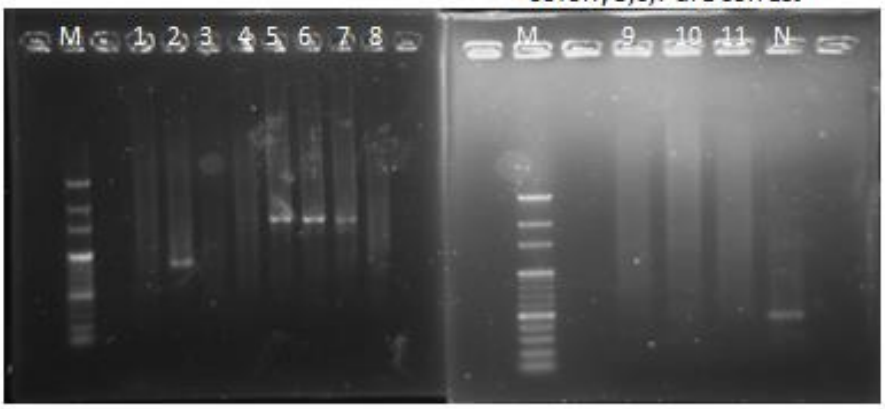
J23100+yebF+endolysin+B0015 (colony PCR)

Content :

1~12=PCR product from colony1~11
- -template=J23100+yebF+endolysin+B0015
- -primer=VF2+VR
- -program=2000TD
- -should be 1434bp

M = 100 bp marker
N = negative control(PCR mix)

Result :
Contamination
Colony 5,6,7 are correct



10/5

J23100+yebF+endolysin+B0015 (plasmid PCR&EP check)

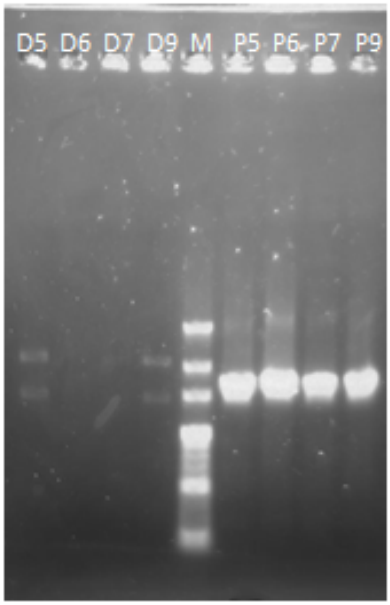
Content :

P5,6,7,9=PCR product from plasmid 5,6,7,9
- -template=J23100+yebF+endolysin+B0015
- -primer=VF2+VR
- -program=2000TD
- -should be 1716 bp

D5,6,7,9=Digestion product
- -template=plasmid 5,6,7,9
- -enzyme=EcoRI & Pst1

M = 100 bp marker

Result :
Contamination
Colony 5,6,7 are correct



10/6

Functional Test:(8/28~10/5)

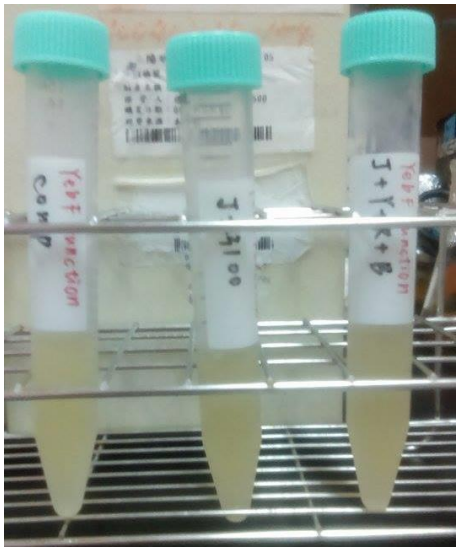
Outline:

1. To test if yebF works, we decide to make comparison within three bacterial culture supernatant :
   J23100+B0034+RFP+B0015
   J23100+RBS-yebF+RFP+B0015
   Competent cell
   By measuring their fluorescent intensity in the range of RFP wavelength in their supernatant, we can conclude whether our YebF function as carrier to deliver RFP outside the cell.
2. Testing if the endolysin works, we use disk plate assay :
   If the content we put on the disk works, there will be an obvious clear trace on S.Mutans plate.

Process:

1. yebF functional test:

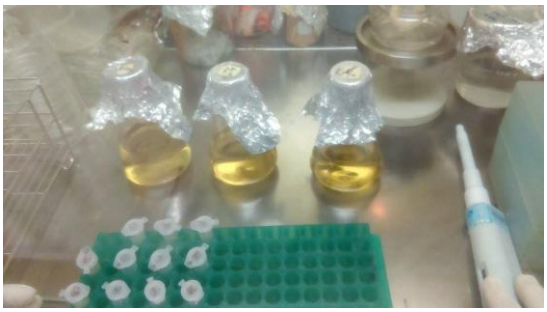
Pre preparation :



From left to right :

- Competent cell
- J23100+B0034+RFP+B0015
- J23100+RBS-yebF+RFP+B0015

The OD_{600} values of the three overnight culture without adding antibiotics are adjusted to 0.6. And by adding 1ml of those cultures into 80ml flask containing LB, we start the culture from 0 hour.



0 hour :

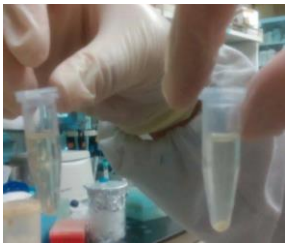
From left to right :

- Competent cell
- J23100+B0034+RFP+B0015
- J23100+yebF+RFP+B0015

Every time we harvest the culture, we add 1c.c. of each of them to two eppendorfs.

One set is for the purpose of growth measurement, while another set is for testing fluorescent intensity of the supernatant. Also, we filled the other set of eppendorfs with pure LB as control.

And for the set of sample which intended to be used in testing fluorescence, we centrifuge them at the speed of 2.0 rcf, for 7 minutes, as the RFP commercial separating kit indicates.



The 4th hour incubation in 37C:

After centrifugation.

The left one : J23100+yebF+RFP+B0015

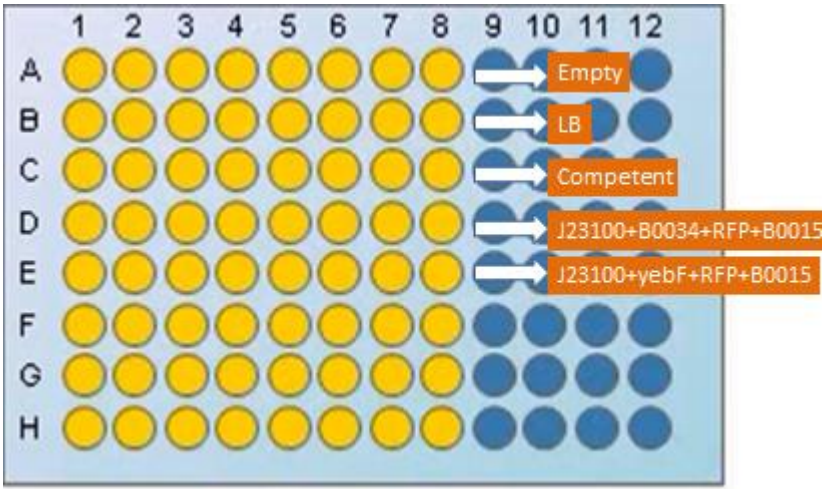
The right one : J23100+B0034+RFP+B0015

After centrifugation, the supernatant were taken out to another Eppendorf.

The next step, we add 200 ul of contents in each eppendorfs to 96 well plate.

By using i-control software in Infinite200 Multimode microplate reader(TECAN). We obtain their OD_{600} absorption value and fluorescence intensity value.

Here is Infinite200 Multimode microplate reader we use, and how we arrange our 96-well plate



What the software looks like:

Application: Tecan i-control Tecan i-control , 1.9.17.0

Device: infinite 200Pro	Serial number: 1201004199	Serial number of connected stacker:
Firmware: V_3.22_12/10_Infinite (Dec 14 2010/13.07.14)	MAI, V_3.22_12/10_Infinite (Dec 14 2010/13.07.14)	

Date: #########
Time: 上午 04:58:38

System	TECAN-THINK
User	Tecan-THINK\Tecan
Plate	ThermoFischer Scientific-Nunclon 96 Flat Bottom Black [NUN96fb.pdfx]
Plate-ID (Stacker)	

Shaking (Orbital) Duration:	10 s
Shaking (Orbital) Amplitude:	1 mm

Label: Label1

Mode	Fluorescence Top Reading	
Excitation Wavelength	584	nm
Emission Wavelength	607	nm
Excitation Bandwidth	9	nm
Emission Bandwidth	20	nm
Gain	89	Optimal (100%)
Number of Flashes	10	
Integration Time	20	µs
Lag Time	0	µs
Settle Time	0	ms
Z-Position (Manual)	20000	µm
Part of Plate	A1-E4	

Start Time: 2014/10/12 上午 04:59:05

Temperature: 28 °C

<>	1	2	3	4

A	41455	42017	44644	43958
B	16782	16284	17359	16695
C	16567	17394	16661	16848
D	16003	15923	17213	16445
E	16544	17437	17424	17285

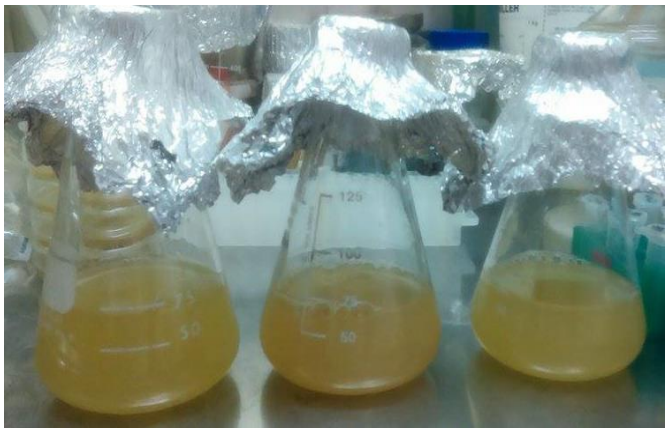
End Time: 2014/10/12 上午 04:59:17

Movement	Move Plate Out

Just as the steps decribed, we did measurement for 20 hours, till we are sure that fluorescence intensity was quite stable.

Here are some note of our process:

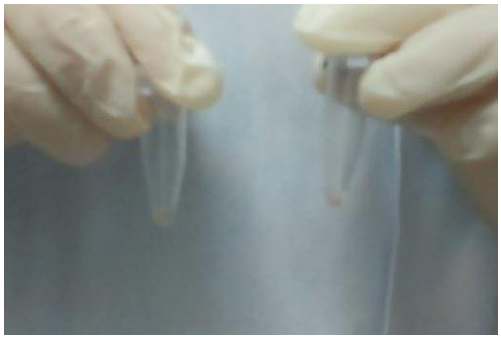
14 hr :



The 14th incubation:

Left to right:

- Competent cell
- J23100+B0034+RFP+B0015
- J23100+yebF+RFP+B0015



The 14th incubation:

After centrifugation

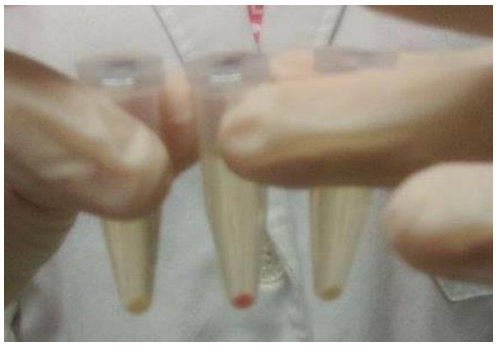
Left to right:

- J23100+B0034+RFP+B0015
- J23100+yebF+RFP+B0015

The 16th incubation:

Left to right:

- Competent cell
- J23100+B0034+RFP+B0015
- J23100+yebF+RFP+B0015



The 16th incubation:

After centrifugation

Left to right:

- Competent cell
- J23100+B0034+RFP+B0015
- J23100+yebF+RFP+B0015

The 16th incubation:

Left to right:

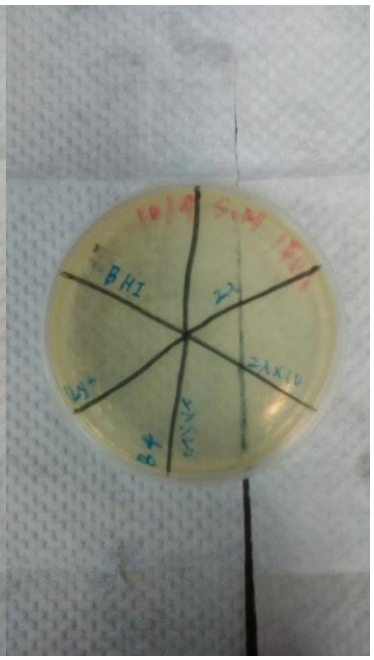
- Competent cell
- J23100+B0034+RFP+B0015
- J23100+yebF+RFP+B0015

2. endolysin functional test :

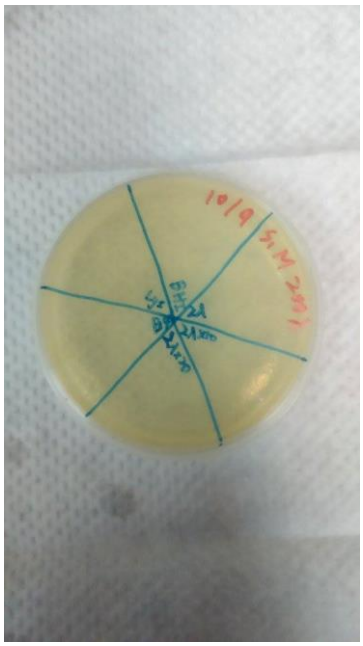
In order to determine how much bacterial culture to put on the BHI plate to gain the most full and uniform Streptococcus Mutans plate. We first take 50ul, 100ul, 150ul, 200ul, and 250ul of S.Mutans two days ago, which has OD_{600} value=1.714 and coated them on BHI plate:

50 lambda

100 lambda



150 lambda



200 lambda

Observing the growth of Streptococcus Mutans on the plate, we have two improvements to be made:

- Adding BHI to dilute the culture in order to have a more uniform coating of plate.
- Use hands while coating the plate instead of turntable.

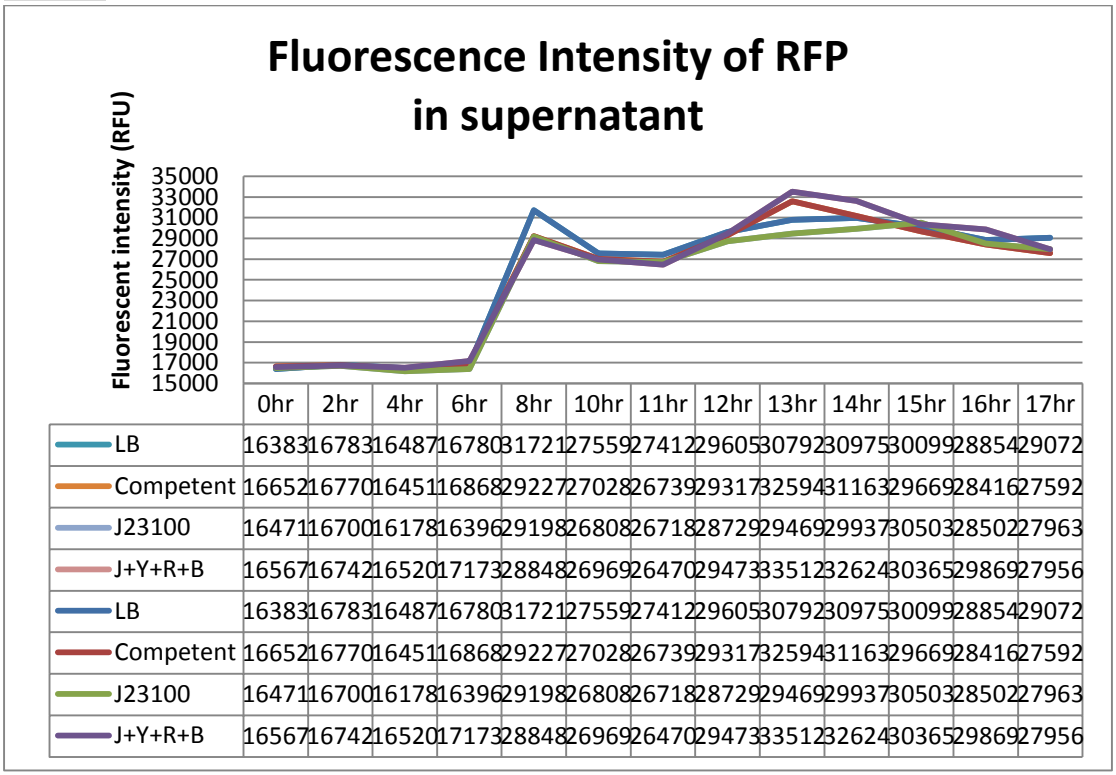
As for the disk to be used on the plate, we use those filter designed for western

blot to cut out small round fragments and autoclave them by putting them on foil paper on yellow tip box.

For the endolysin and control circuit supernatant, we obtained those from overnight culture of those E.coli. Take 500ul of them and measure OD_{600} value to estimate an approximate bacterial amount. Adjusting Testing and Controlling culture to the same value by dilution of BHI, we took them to centrifuge (speed 2.0 rcf for 7 minutes, as the size is about the same with RFP) and collect the supernatant.

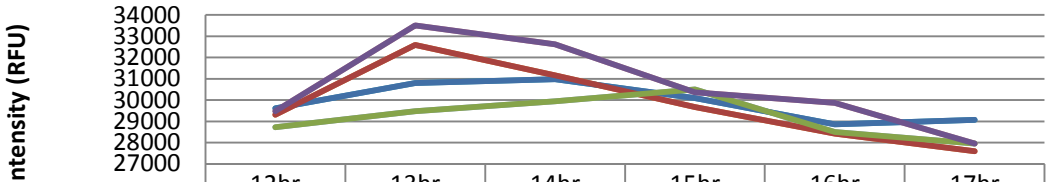
Adding positive control (lysozyme/antibiotics), negative control(BHI), and different amount of the endolysin supernatant, on filter paper, we observe S.Mutans growth near them.

Result :



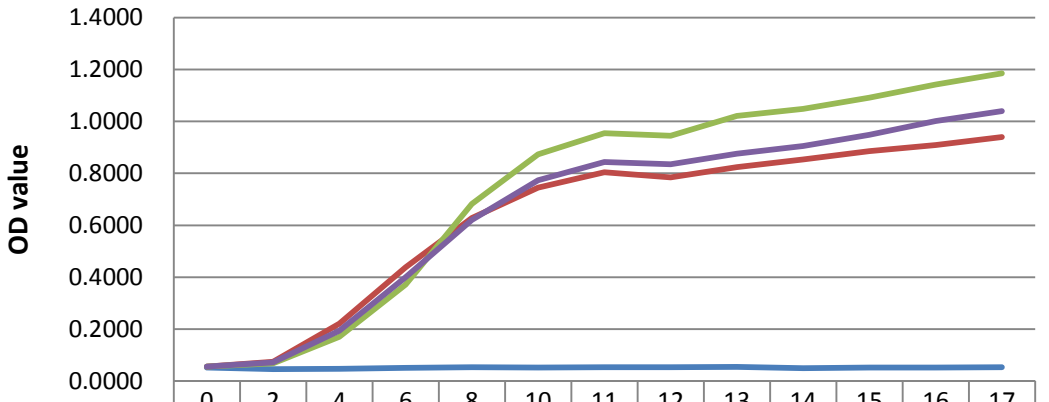
	0hr	2hr	4hr	6hr	8hr	10hr	11hr	12hr	13hr	14hr	15hr	16hr	17hr
LB	16383	16783	16487	16780	31721	27559	27412	29605	30792	30975	30099	28854	29072
Competent	16652	16770	16451	16868	29227	27028	26739	29317	32594	31163	29669	28416	27592
J23100	16471	16700	16178	16396	29198	26808	26718	28729	29469	29937	30503	28502	27963
J+Y+R+B	16567	16742	16520	17173	28848	26969	26470	29473	33512	32624	30365	29869	27956
LB	16383	16783	16487	16780	31721	27559	27412	29605	30792	30975	30099	28854	29072
Competent	16652	16770	16451	16868	29227	27028	26739	29317	32594	31163	29669	28416	27592
J23100	16471	16700	16178	16396	29198	26808	26718	28729	29469	29937	30503	28502	27963
J+Y+R+B	16567	16742	16520	17173	28848	26969	26470	29473	33512	32624	30365	29869	27956

Fluroescence Intensity of RFP in supernatant_Partial Enlargement

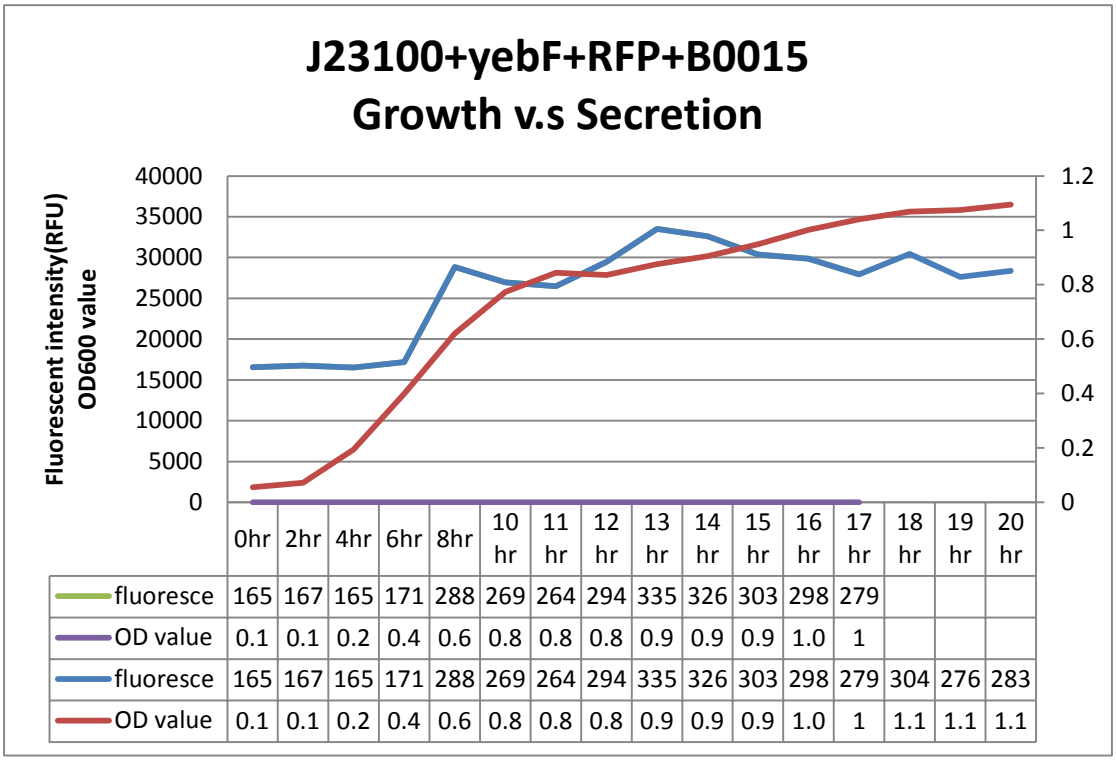


	12hr	13hr	14hr	15hr	16hr	17hr
LB	29604.5	30792	30975.25	30098.75	28853.75	29071.5
Competent	29316.5	32594	31163.25	29668.5	28416.25	27591.75
J23100	28728.75	29469.25	29937.25	30503	28502	27963
J+Y+R+B	29473.25	33512.25	32623.5	30365.25	29868.5	27956
LB	29604.5	30792	30975.25	30098.75	28853.75	29071.5
Competent	29316.5	32594	31163.25	29668.5	28416.25	27591.75
J23100	28728.75	29469.25	29937.25	30503	28502	27963
J+Y+R+B	29473.25	33512.25	32623.5	30365.25	29868.5	27956

growth curve



	0	2	4	6	8	10	11	12	13	14	15	16	17
LB	0.052	0.046	0.047	0.051	0.053	0.052	0.053	0.053	0.054	0.05	0.052	0.051	0.053
Comp	0.056	0.074	0.221	0.437	0.627	0.745	0.805	0.784	0.824	0.854	0.885	0.909	0.939
J23100	0.056	0.068	0.17	0.372	0.682	0.874	0.955	0.945	1.021	1.048	1.092	1.142	1.185
J+Y+R+B	0.056	0.072	0.194	0.399	0.621	0.774	0.844	0.835	0.876	0.906	0.949	1.001	1.04



	0hr	2hr	4hr	6hr	8hr	10 hr	11 hr	12 hr	13 hr	14 hr	15 hr	16 hr	17 hr	18 hr	19 hr	20 hr
fluoresce	165	167	165	171	288	269	264	294	335	326	303	298	279			
OD value	0.1	0.1	0.2	0.4	0.6	0.8	0.8	0.8	0.9	0.9	0.9	1.0	1			
fluoresce	165	167	165	171	288	269	264	294	335	326	303	298	279	304	276	283
OD value	0.1	0.1	0.2	0.4	0.6	0.8	0.8	0.8	0.9	0.9	0.9	1.0	1	1.1	1.1	1.1